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Analysis of benidipine enantiomers in human plasma by liquid chromatography–mass spectrometry using a macrocyclic antibiotic (Vancomycin) chiral stationary phase column

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Abstract

We used a novel chromatographic method to rapidly and simply characterize the pharmacokinetics of benidipine enantiomers in human plasma. The stereoisomers of benidipine were extracted from plasma using diethylether under alkaline conditions. After evaporating the organic layer, the residue was reconstituted in the mobile phase (methanol:acetic acid:triethylamine, 100:0.01:0.0001, v/v/v). The enantiomers in the extract were separated on a macrocyclic antibiotic (Vancomycin) chiral stationary phase column. The mobile phase was eluted at 1 ml/min and was split by an interface. One-fifth of the eluent was used to quantify both isomers in a tandem mass spectrometer in multiple reaction-monitoring mode. The coefficient of variation of the precision of the assay was less than 8%, the assay accuracy was between 93.4 and 113.3%, and the limit of detection was 0.05 ng/ml for 1 ml of plasma. The method described above was used to measure the concentration of both benidipine enantiomers in plasma from healthy subjects who received a single oral dose of a racemate of 8 mg benidipine. The C_{max} and AUC_{inf} values of (+)-alpha benidipine were higher than those of (−)-alpha benidipine by 1.96- and 1.85-fold, respectively ($p < 0.001$), whereas, the T_{max} and $t_{1/2}$ for each of the benidipine stereoisomers were not significantly different.

Keywords: Benidipine; Enantiomers; Chiral stationary phase; Tandem mass spectrometry

1. Introduction

Benidipine is a dihydropyridine calcium antagonist that is used clinically as a racemate, containing the (−)-alpha and (+)-alpha isomers of benidipine. In animal studies, the kinetic behavior and dynamic efficacies, respectively, of the benidipine stereoisomers were different [1–4]. Because the hepatic intrinsic clearance rate for (−)-alpha benidipine is greater than that for (+)-alpha benidipine, plasma concentrations of (+)-alpha benidipine are greater than those of (−)-alpha benidipine following the administration of a benidipine racemate to rats [5]. In addition, the hypotensive effect of (+)-alpha benidipine was between 30- and 100-fold the effect of (−)-alpha benidipine in spontaneously hypertensive rats [1]. The aforementioned observations suggest that the competitive metabolism of the stereoisomers of benidipine that follows the oral administration of the benidipine racemate gives rise to differential pharmacokinetics for the different enantiomers of benidipine.

Although several different methods have been used to measure benidipine concentrations in plasma [6–9], the enantioselective pharmacokinetics of the stereoisomers of benidipine in humans has not been examined. Therefore, we developed a novel chiral chromatography method to determine

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the enantioselective pharmacokinetics of benidipine in human plasma.

In general, the chiral resolution of stereoisomers has been carried out using (i) a specific chiral stationary phase, (ii) a mobile phase with a chiral reagent, and (iii) a derivatization method [10], and many types of chiral chromatography columns are available commercially. In the present study, we introduce a rapid and simple chromatographic method to separate the (+)-alpha and (−)-alpha isomers of benidipine. The method is based on the use of a chiral column and a tandem mass spectrometer. We used the method successfully to characterize the time course of changes in the plasma concentrations of the benidipine stereoisomers in human blood plasma following the oral administration of a benidipine racemate.

2. Experimental

2.1. Reagents and materials

The benidipine enantiomers (+)-(*R**)-2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid (*R**)-1-benzyl-3-piperidinyl ester, methyl ester hydrochloride [the (+)-alpha isomer] and (−)-(*R**)-2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid (*R**)-1-benzyl-3-piperidinyl ester, methyl ester hydrochloride [the (−)-alpha isomer], as well as benidipine-d_5 hydrochloride (the internal standard, IS) were kindly donated by Kyowa Hakko Kogyo Co. Ltd. (Shizuoka, Japan; Fig. 1). High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of the highest analytical grade available.

2.2. Preparation of standards and quality controls

The benidipine isomers and the IS were dissolved in methanol to obtain a concentration of 1.0 and 0.1 mg/ml, respectively. The aforementioned solutions were diluted serially with methanol before being added to drug-free plasma to obtain final concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/ml. Using linear regression, two calibration graphs were derived from the ratio between the area under the peak of each isomer and the IS (which was eluted in advance).

Quality control samples were prepared in 1 ml of blank human plasma by adding 100 μl of the serial dilution solution of each benidipine isomer to obtain minimum (0.2 ng/ml), intermediate (0.5 and 1.0 ng/ml), and high (5 and 10 ng/ml) concentration control samples. These samples were used to evaluate the inter- and intra-day precision and accuracy of the assay.

2.3. Characterization of tandem mass spectrometry product ions

We used the same conditions for the mass spectrometry as those described previously [9]. Briefly, 1 μM each of the benidipine isomer and IS solutions was infused into the mass spectrometer separately at a flow rate of 10 μl/min to characterize the product ions of each solution. The precursor ions $[M+H]^+$ and the pattern of fragmentation were monitored using the positive ion mode. The major peaks in the MS/MS

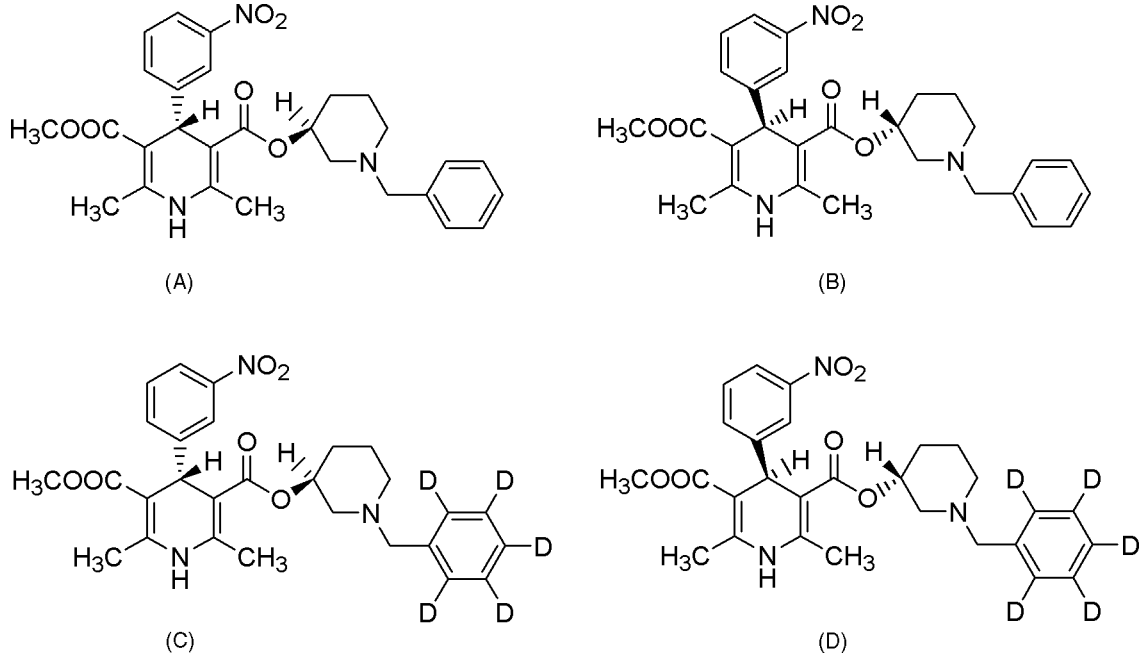

Fig. 1. Structures of benidipine enantiomers (A) (+)-alpha benidipine, (B) (−)-alpha benidipine and internal standard, (C) (+)-alpha benidipine-d_5, (D) (−)-alpha benidipine-d_5.

scan were used to quantify the benidipine isomers and the IS.

2.4. Analytical system

Plasma benidipine concentrations were quantified using liquid chromatography–mass spectrometry with an API 3000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface that was used to generate positive ions ($[M+H]^+$). Compounds were separated on a chiral stationary phase column (Chirobiotic V, 150 mm × 4.6 mm internal diameter, 5 μm particle size; Advanced Separation Technologies, Whippany, NJ, USA) with an isocratic mobile phase consisting of 10^{-2}% acetic acid and 10^{-4}% triethylamine in methanol. The column was heated to 40 °C, and the mobile phase was eluted at 1 ml/min using an HP 1100 series pump (Agilent, Wilmington, DE, USA). The mobile phase was divided into two parts (0.8 ml/min was diverted to waste and 0.2 ml/min was used for quantification) using a splitter that was located between the column and the tandem mass spectrometer.

The Turboion spray interface was operated in the positive ion mode at 5500 V at 350 °C. The operating conditions were optimized by flow-injecting a mixture of all analytes and were as follows: nebulizing gas flow, 1.04 l/min; auxiliary gas flow, 4.0 l/min; curtain gas flow, 1.44 l/min; orifice voltage, 80 V; ring voltage, 400 V; and collision gas (nitrogen) pressure, 3.58×10^{-5} Torr. Quantitation was performed by multiple reaction-monitoring (MRM) of the protonated precursor ions and the related product (benidipine) ions using the ratio of the area under the peak for each solution and a weighting factor of $1/x$. Since, the weighting factor reduces, the bias from the upper concentration and decreases the error at the lower one, it provided a more accurate prediction due to a better correlation between concentration and the ratio. A further weighting factor of $1/x^2$ could not give us a lower Akaike's information criterion [11]. Therefore, the former weighting factor was finally chosen. The mass transition for benidipine and the IS was m/z 506 → 174 and 511 → 179, respectively (collision energy, 36 eV; dwell time, 200 ms). Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed with Analyst software (version 1.2, Applied Biosystems, Foster City, CA, USA).

2.5. Sample preparation

Twenty microliters of the IS (100 ng/ml) and 1 ml of 5 N NaOH were added to 1 ml of plasma, followed by liquid–liquid extraction for 1 min with 5 ml diethylether. The organic layer was separated and removed at ambient temperature in a heated centrifugal evaporator (Speed-Vac; Savant, Holbrook, NY, USA). The residue was reconstituted into 100 μl of the mobile phase by vortex-mixing for 15 s; 50 μl of this solution was then injected onto the column. After splitting the eluate, a final volume of 10 μl of the injectate was processed in the tandem mass spectrometer.

2.6. Validation procedure

The validation parameters were selectivity, extraction recovery, precision, and accuracy. Blank plasma samples obtained from 10 volunteers were screened to determine specificity. The extraction recoveries of benidipine were calculated by comparing the peak area ratios measured for the standard solution considering condensation with those obtained for plasma extracts after the extraction procedure. The intra- and inter-day assay precision and accuracy were estimated by using the calibration curve to predict the concentration of the quality controls. For the stability study in plasma, control drug-free plasma samples were spiked with 0.5 and 5 ng/ml benidipine. Short-term stability was assessed after 12 h of storage at room temperature; long-term stability was assessed after 4 weeks of storage in a freezer at −80 °C. The stability of benidipine in plasma samples was tested after three freeze–thaw cycles (−80 °C to room temperature). The stability of benidipine in extracts was also examined after 10 h of storage at room temperature.

2.7. Clinical application

Ten healthy subjects who provided written informed consent took part in the study. Health problems, drug or alcohol abuse, and abnormalities in standard laboratory screening test results were exclusion criteria. This study was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea). After an overnight fast, all subjects were given a single oral dose of 8 mg benidipine, a mixture of 4 mg of (+)- and (−)-alpha isomer, respectively. Blood (6 ml) was withdrawn before and at 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, 4.00, 5.00, 6.00, and 8.00 h after drug administration. Plasma was separated following centrifugation at 1000 × g for 10 min and was stored at −80 °C until analyzed.

2.8. Pharmacokinetic analysis

The pharmacokinetic analysis of the benidipine isomers was performed using non-compartmental methods. The area under the plasma concentration versus time curves (AUC) calculated using the trapezoidal rule and was extrapolated to infinity. The time course of plasma benidipine concentrations was used to determine the maximum plasma concentration (C_{max}) and the time (T_{max}) to reach C_{max}. The elimination rate constant (k_{el}) was obtained by linear regression of the terminal phase, and the calculated elimination half-life ($t_{1/2}$) was $0.693/k_{el}$. Statistical significance of various pharmacokinetic parameters between two enantiomers were analyzed by a paired t-tests and $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Chiral separation

The chromatographic resolution of benidipine enantiomers was accomplished directly on the Chirobiotic V analytical column. The chiral stationary phase column is based on bonding to Vancomycin, which contains 18 chiral centers that surround three pockets or cavities; five aromatic ring structures bridge these strategic cavities, and hydrogen donor and acceptor sites are readily available close to the ring structures. The Chirobiotic V column exhibits selectivity that is similar to that of glycoprotein phases [12].

The (−)-alpha and (+)-alpha benidipine isomers were eluted at 2.4 and 3.4 min, respectively, with a satisfactory resolution, and benidipine-d_5 (the IS) was separated into two peaks at 2.5 and 3.5 min (Fig. 2). The column temperature could not be elevated above 40 °C unlike the case for the chiral separation of manidipine (50 °C) [10], because the maximum column temperature that can be employed safely is 45 °C. In some compounds, two effects were observed at elevated temperatures: an increase in efficiency and/or a reversal of the elution order. The temperature that we selected was sufficient to achieve good separation without reversal.

The composition of the mobile phase was selected from the previous literatures, which used the same column [13,14]. Additives of both acetic acid and triethylamine were necessary to successfully separate benidipine isomers. One percentage of triethylamine was added at methanol, including acetic acid to optimize the chromatographic condition at the very beginning and its amount was gradually reduced to minimize a contamination due to unvolatile property, and finally decided at 0.01%. Such a small amount of triethylamine should be still necessary to separate benidipine enantiomers.

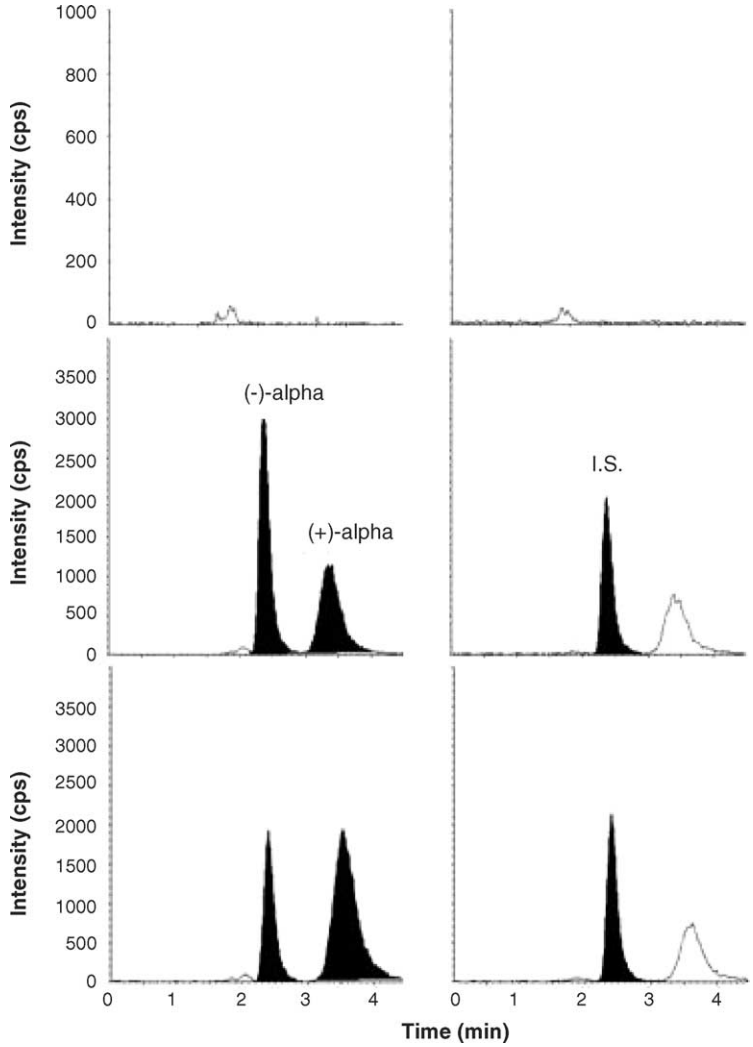

Fig. 2. Chromatograms of benidipine enantiomers (left) and benidipine-d_5 (right). Top, blank plasma; middle, plasma spiked with 1 ng/ml (−)- and (+)-alpha benidipine and benidipine-d_5; bottom, plasma sample equivalent to 0.76 ng/ml for (−)-alpha benidipine and 1.47 ng/ml for (+)-alpha benidipine in a sample obtained from a volunteer at 2 h after oral administration of 8 mg benidipine.

Table 1
Precision and accuracy of the intra-day assay of benidipine enantiomers ($n = 6$)

Added (ng/ml)	(−)-Alpha benidipine		(+)-Alpha benidipine	
	Accuracy (mean% ± S.D.)	R.S.D. (%)	Accuracy (mean% ± S.D.)	R.S.D. (%)
0.2	106.5 ± 8.5	8.0	103.3 ± 7.6	7.4
0.5	106.8 ± 6.8	6.4	97.3 ± 6.3	6.5
1.0	97.4 ± 0.3	0.3	104.3 ± 3.2	3.0
5.0	97.4 ± 0.5	0.5	98.3 ± 1.1	1.1
10.0	93. 4 ± 0.9	0.9	104.4 ± 3.4	3.2

R.S.D. (relative standard deviation, %) = S.D. × 100/mean.

3.2. Quantification of benidipine and validation of the method

There were no interfering peaks at the elution times for either of the benidipine isomers or the IS. Fig. 2 presents typical chromatograms for blank plasma (top), plasma spiked with both compounds (1 ng/ml of each benidipine isomer and 100 ng/ml of IS) (middle), and a plasma sample from a volunteer (bottom).

The calibration curves provided a reliable response for benidipine concentrations between 0.05 and 10.00 ng/ml. The ratio of the area of the peak of each benidipine isomer relative to that of the IS (which was eluted in advance) was correlated with the corresponding plasma concentration, and good linearity was observed. The equations of the regression lines for (−)-alpha and (+)-alpha benidipine were $y = (1.094 \pm 0.046)x - (0.021 \pm 0.075)$, ($r^2 > 0.999 \pm 0.002$), and $y = (1.543 \pm 0.026)x - (0.005 \pm 0.024)$, ($r^2 > 0.999 \pm 0.001$), respectively. The detection limit for both benidipine isomers was 0.05 ng/ml at a signal-to-noise (S/N) ratio of 3.

The estimates of intra- and inter-day precision and accuracy of the assay are presented in Tables 1 and 2, respectively. The relative standard deviation (R.S.D.) of the intra-day assay precision were less than 8.0% and 7.4% for (−)-alpha and (+)-alpha benidipine, respectively. The accuracies of the intra-day assay were 93.4–106.8% and 97.3–104.3% for (−)-alpha and (+)-alpha benidipine, respectively. The R.S.D.s of the inter-day assay precision were less than 7.1% and 7.7% for (−)-alpha and (+)-alpha benidipine, respectively. The accuracies of the inter-day assay were 94.7–110.2% and 94.8–113.3% for (−)-alpha and (+)-alpha benidipine, respectively. The mean recovery of both isomers for benidipine concentrations ranging from 0.05 to 10.00 ng/ml exceeded 90% for both the intra- and inter-day assays. Benidipine was stable in plasma at room temperature for up to at least 12 h; it also remained intact at −80 °C for up to 4 weeks. With respect to the run-time stability of processed samples, no significant loss of benidipine was observed at room temperature, and no degradation was observed after three cycles of freezing and thawing (Table 3). This information provides confidence to perform repeated analyses of clinical samples within 12 h.

3.3. Application of the method

The validated method (described above) was used to evaluate the enantioselective pharmacokinetics of benidipine in human blood plasma. Fig. 3 shows the mean plasma concentrations of (+)-alpha and (−)-alpha benidipine in plasma after a single oral dose of 8 mg benidipine. The pharmacokinetic parameters of both benidipine isomers are presented in Table 4. The plasma concentrations of (+)-alpha benidipine were always greater than those of (–)-alpha benidipine. The C_{max} and AUC_{inf} values of (+)-alpha benidipine (1.47 ± 0.75 ng/ml and 2.48 ± 1.18 ng h/ml, respectively) were higher than those of (−)-alpha benidipine (0.75 ± 0.46 ng/ml and 1.34 ± 0.48 ng h/ml, respectively) by 1.96- and 1.85-fold, respectively ($p < 0.001$). The T_{max} and $t_{1/2}$ for each of the benidipine stereoisomers were not significantly different: (−)-alpha and (+)-alpha benidipine reached a peak concentration at 0.85 ± 0.29 h and 0.71 ± 0.26 h, respectively, the half-lives at the terminal phase were 0.49 ± 0.10 h and 0.52 ± 0.17 h for (−)-alpha and (+)-alpha benidipine, respectively.

The results of the present study concur with the results of a study of benidipine pharmacokinetics in rat. The higher concentration of the (+)-alpha enantiomer of benidipine in humans that we observed in the present study might be

Table 2
Precision and accuracy of the inter-day assay of benidipine enantiomers ($n = 6$)

Added (ng/ml)	(−)-Alpha benidipine		(+)-Alpha benidipine	
	Accuracy (mean% ± S.D.)	R.S.D. (%)	Accuracy (mean% ± S.D.)	R.S.D. (%)
0.2	110.2 ± 7.5	6.8	113.3 ± 8.6	7.5
0.5	104.5 ± 7.4	7.1	94.8 ± 7.3	7.7
1.0	94.7 ± 2.3	2.4	102.3 ± 4.5	4.4
5.0	98.4 ± 1.6	1.6	99.3 ± 3.1	3.1
10.0	98.8 ± 3.9	3.9	102.3 ± 4.6	4.5

R.S.D. (relative standard deviation, %) = S.D. × 100/mean.

Table 3
Stability of benidipine after storage under indicated condition

Concentration (ng/ml)	Storage condition			
	Ambient in extracts (10 h)	Ambient (12 h)	−80 °C (4 weeks)	Three cycles of freezing–thawing
0.5	0.49 ± 0.02[a]	0.48 ± 0.03	0.48 ± 0.05	0.53 ± 0.03
5.0	5.03 ± 0.04	4.95 ± 0.07	4.89 ± 0.12	5.10 ± 0.09

[a] Mean ± standard deviation ($n = 3$).

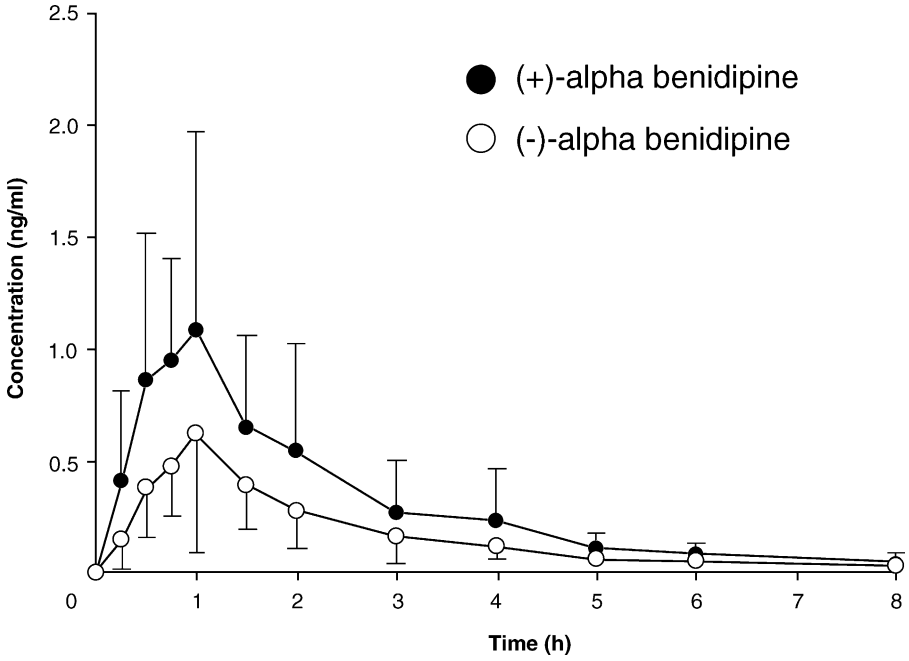

Fig. 3. Time course of the concentration of benidipine enantiomers in plasma samples obtained from healthy subjects ($n = 10$) after a single oral dose of 8 mg benidipine. Each point represents the mean ± S.D.

attributable to the prior stereoselective metabolism of (−)-alpha benidipine by microsomal enzymes, as is the case in rat [5]. The competitive inhibition of the metabolism of the different benidipine enantiomers in humans remains to be investigated.

In conclusion, a stereoselective and highly sensitive method to determine the pharmacokinetics of benidipine enantiomers in human plasma was developed by combining separation via a chiral stationary phase column with tandem mass spectrometry. This method is suitable for clinical pharmacokinetic studies of benidipine enantiomers after the oral administration of a benidipine racemate.

Table 4
Pharmacokinetic parameters[a] (mean ± S.D.) of benidipine enantiomers in plasma from 10 healthy subjects after a single oral dose of 8 mg benidipine

Parameter	(−)-Alpha benidipine	(+)-Alpha benidipine	p
C_{max} (ng/ml)	0.75 ± 0.46	1.47 ± 0.75	<0.001
T_{max} (h)	0.85 ± 0.29	0.71 ± 0.26	NS
AUC_{inf} (ng h/ml)	1.34 ± 0.48	2.48 ± 1.18	<0.001
$t_{1/2}$ (h)	0.49 ± 0.10	0.52 ± 0.17	NS

NS: no statistical significance.
[a] See main text for parameter definitions.

Acknowledgments

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